Quantitative Comparison of Selected Virulence Associated Characteristics in Food and Clinical Isolates of *Listeria*

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ABSTRACT

Thirty food and clinical isolates of *Listeria* were compared quantitatively in regard to lethality in immunocompromised mice, hemolytic activity for sheep erythrocytes, invasiveness towards Hep-2 epithelial cells, and cytotoxicity to CHO cells. All *Listeria monocytogenes* isolates were hemolytic, invasive, weakly cytotoxic, and lethal to immunocompromised mice. *Listeria ivanovii* isolates expressed the first three properties but were nonvirulent. There was little quantitative correlation among the virulence markers, suggesting that there may be additional virulence related factors that may influence the pathogenicity of *L. monocytogenes* isolates. No systematic differences between the clinical and food isolates were apparent. Electron and light microscopy of infected Hep-2 cells revealed *L. monocytogenes* and *L. ivanovii* encapsulated within cell processes containing an actin matrix.

Listeria monocytogenes is a gram-positive psychrotrophic bacterium that causes perinatal infections, septicemia, and meningo-encephalitis in humans and several animal species (*8,9*). Recent outbreaks have emphasized the importance of foods in the etiology of epidemic listeriosis (*4,12,19,30*). The intestinal epithelium is the usual site of entry of this microorganism (*26,27*), and since low levels of *L. monocytogenes* in fresh products of animal origin are common (*2*), there is a need to predict the potential virulence of foodborne isolates.

Hemolytic activity correlates qualitatively, but not quantitatively, with virulence for mice (*10,14,29*). Additional in vitro tests that have been used to qualitatively assess the virulence of *Listeria* isolates include (a) cytotoxic effects on continuous cell lines and peritoneal exudate cells (*3,24,32*); (b) invasion to epithelial and fibroblast cells (*6,11,18,25,26,27*); and (c) ability to multiply within phagocytic cells (*5,20,21*). However, there have been few studies that have examined the quantitative relationships among these virulence associated markers. Accordingly, the objective of the current study was to determine the quantitative relationships between lethality in immunocompromised mice, invasiveness to cultured epithelial cells, hemolytic activity, and cytotoxicity, with particular emphasis on determining if there are any systematic differences between food and clinical isolates of *Listeria* spp.

MATERIALS AND METHODS

Bacteria

The origin and relevant characteristics of the bacterial strains used in this study are summarized in Table 1. Stock cultures were maintained in tryptose phosphate agar (TPA) (Difco Laboratories, Detroit, MI) at 4°C. Working cultures were kept on TPA at room temperature.

Invasive assay

Hep-2 cell monolayers were grown and maintained at 37°C under 5% CO_2 and humid air using Dulbecco's modification of Eagle minimal essential medium [(DMEM), Flow Labs, McLean, VA] supplemented with 10 mM L-glutamine and 10% fetal bovine serum. For the assay, cells were trypsinized, suspended in DMEM, and seeded [1 ml of cells (5×10^5 cells/ml)] into 24 well flat bottom tissue culture plates [17 x 1.6 cm wells, (Flow Labs)]. Cells were incubated for 2-3 d to obtain a semiconfluent monolayer. Bacterial cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of tryptose phosphate broth (TPB) for 24 h at 37°C using a shaker incubator (150 rpm). Bacterial suspensions were washed three times in DMEM, adjusted turbidimetrically (OD 1.5 660 nm = 3.0×10^9 CFU/ml) to approximately 1.0×10^7 CFU/ml in DMEM, and 1.0 ml added to each of three wells containing Hep-2 cells. Colony counts were performed to determine the multiplicity of infection (MOI) which was approximately 10:1. All monolayers were infected for 1 h at 37°C under 5% CO_2, and then washed three times in DMEM containing 100 µg/ml of gentamycin sulphate. An exception were the monolayers (designated T_0, extra and intracellular), used to determine the initial level of bacteria which were washed in DMEM without gentamycin. The remaining monolayers were overlaid with 2.0 ml of the antibiotic containing medium and left for the duration of the experiment to kill extracellular bacteria (*25*). The subsequent fate of intracellular bacteria was determined by further incubation for 1 (T_1) and 3 h (T_3) under the same conditions.

Intracellular bacteria were released by first washing cell monolayers three times with cold DMEM without gentamycin, followed by addition of 2 ml of sterile distilled water containing 1% Triton X-100. The lysate was plated in duplicate onto TPA plates with a spiral plater (Spiral Systems, Inc., Cincinnati, OH) and incubated for 48 h at 37°C. The suspending medium (DMEM plus antibiotics) was routinely cultured to monitor the presence of exocytosed and/or free bacteria not killed by the antibiotic,

TABLE 1. Listeria *strains and their sources.*

Strain	Species	Source
P5-VJ-G	*innocua*	pork sausage (USDA)
LA-1	*innocua*	cheese (FDA)
SA3-VT	*innocua*	salami (USDA)
SS-L-S	*innocua*	shrimp salad (USDA)
LG5-LS	*innocua*	ground lamb (USDA)
H2-LG	*innocua*	hamburger (USDA)
SH3-VJ-G	*innocua*	shrimp (USDA)
KCL-1714	*ivanovii*	(CDC)
F-5999	*ivanovii*	(CDC)
F-4080	*selligeri*	(CDC)
GVL4-VS	*welshimeri*	ground veal (USDA)
GVL4-LS	*welshimeri*	ground veal (USDA)
H2-VJ-g	*welshimeri*	hamburger (USDA)
CF1-VP	*welshimeri*	chicken (USDA)
GV2-VS	*monocytogenes* (1)[1]	ground veal (USDA)
S9-VJ-G	*monocytogenes* (1)	sausage (USDA)
H4-V-G	*monocytogenes* (1)	hamburger (USDA)
Scott-A	*monocytogenes* (4)	clinical (CDC)
HO-VJ-G	*monocytogenes* (1)	hamburger (USDA)
MF2-L-P	*monocytogenes* (1)	fish (USDA)
GVN4-VG	*monocytogenes* (1)	ground veal (USDA)
GLBI-LS	*monocytogenes* (1)	ground lamb (USDA)
V3-VT	*monocytogenes* (1)	veal patties (USDA)
LG4-VS	*monocytogenes* (1)	ground lamb (USDA)
GVG-VS	*monocytogenes* (1)	ground veal (USDA)
CCR8-V-G	*monocytogenes* (1)	chicken patties (USDA)
F-4259	*monocytogenes* (1)	clinical (CDC)
F3-VJ-G	*monocytogenes* (1)	fish (USDA)
VS2-VJ	*monocytogenes* (1)	veal sausage (USDA)
15313	*monocytogenes* (NT)	nonhemolytic (ATCC)

[1](Serotype).
(NT) not typeable.

and the survivors were subtracted from the total plate counts from corresponding lysate. Total plate counts were used to calculate the infectivity of *Listeria* spp.

Cytotoxicity assay

Chinese hamster ovary (CHO) cell monolayers were maintained in HAMS F-12 medium (Flow Laboratories, McLean, VA) supplemented with L-glutamine and 10% fetal bovine serum at 37°C in 5% CO_2 and humid air. For the assay, cells were trypsinized and seeded into 96 flat bottom tissue culture wells [(1.0 x 0.6 cm^2), Flow Labs] with a cell density of 5.0 x 10^5 cell/ml. Confluent monolayers were obtained after 24 h incubation. Bacterial cultures were grown in TPB for 24 h at 37°C in a shaker incubator (150 rpm). Sterile cell-free suspensions were prepared by removing the bacterial cells by centrifugation and then filtering the supernatant through a 0.22 μm membrane filter. Serial dilutions of the suspensions were made in the tissue culture wells using fresh HAMS F-12 medium with and without 10% serum. In addition, 10 ml aliquots of culture filtrates were treated with 100 μl of cysteine hydrochloride (0.01 M, final pH 7.2) for 30 min at 37°C to activate the hemolysin (listeriolysin O). Titers were determined visually after 24 h of incubation at 37°C in 5% CO_2, as the highest dilution showing greater than 50% of the cells affected morphologically.

Hemolysin assay

Hemolytic activity was determined with 1% fresh sheep red blood cells in pH 7.0 phosphate buffered saline (PBS). Bacteria were grown in TPA and cell-free filtrates prepared as described previously. Where appropriate, filtrates were activated by addition of 100 μl of cysteine hydrochloride (0.01 M, pH 7.2) to 1.0 ml of filtrate, followed by incubation for 30 min at 37°C. Hemolytic activity was determined in 96 U bottom well tissue culture plates (Flow Labs). Twofold dilution series were done in PBS with both activated and nonactivated culture filtrates. Equal (100 μl) volumes of filtrate and washed red blood cells were incubated at 37°C for 1 h. Titers were determined visually as the last dilution showing greater than 50% lysis. Uninoculated TPB served as the control.

Mouse lethality

Listeria lethality was determined using 15-20 g Swiss Webster mice which were macrophage-depleted (immunocompromised) by intraperitoneal injection of 200 mg of carrageenan (Type II) in sterile saline (Sigma Chemical Co., St. Louis, MO) per kilogram body weight 24 h before infection (*31*). Bacterial isolates were grown in TPB in a shaker incubator for 24 h at 37°C, washed in PBS, and adjusted to an optical density of 1.5 at 660 nm. Three 100-fold serial dilutions were prepared and five mice were injected intraperitoneally with 0.5 ml of the appropriate dilution. The mice were observed for 10 d and deaths recorded. The 50% median lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (*28*). In each experiment, immunocompromised mice injected with sterile saline served as controls.

Microscopy

For microscopic examination of invasion, Hep-2 cells were cultured in 24 well flat bottom tissue culture plates as indicated earlier with the exception that 12 mm sterile glass coverslips were placed on the bottom of the wells before seeding the cells into the chambers. Bacterial cells [*L. monocytogenes* (Scott-A), *L. ivanovii* (F-5999), and *L. innocua* (H2-LG)] were grown as described previously and the MOI adjusted to approximately 10:1 and 100:1 bacteria/cell. For all microscopic studies, monolayers were infected as previously described, washed three times, and incubated for 3 h in the presence of gentamycin sulphate. For transmission (TEM) and scanning electron microcopy (SEM), coverslips were removed and fixed by immersion in 1% glutaraldehyde in PBS at 37°C for 1-24 h, postfixed in 2% osmium tetraoxide in 0.2 M sodium cacodylate buffer (pH 7.2), and dehydrated in a graded series of ethanol solutions. For final TEM processing, fixed coverslips were embedded in an epoxy resin mixture and thin sections cut, and stained with uranyl acetate and lead citrate solutions. For SEM, fixed coverslips were critical point dried and coated with a thin layer of gold. Specimens were examined using a Zeiss 10B transmission and a JEOL 840A scanning electron microscope.

Fluorescence localization of cellular microfilaments (F-actin) in infected and noninfected monolayers was done fixing the cells by immersing the coverslips in 3.7% formaldehyde in warm PBS followed by permeabilization, by treatment with 0.1% Triton X-100 in PBS. The cells were then stained with fluorescein labeled phalloidin (Molecular Probes, Inc., Eugene, OR) and mounted in 50% glycerol in PBS. Monolayers were also stained with a 1:10 dilution of fluorescein-labeled *Listeria* antisera [Somatic O, 1 & 4 antigens (Difco Labs)]. Microscopy was performed with optics for epifluorescence.

RESULTS

The LD_{50} of all isolates of *Listeria* were estimated in mice immunocompromised by treatment with carrageenan.

TABLE 2. *Mouse lethality and Hep-2 invasive values of* Listeria *spp.*

Strain	Species	*Listeria* (Log CFU) T_0	T_1	T_3	LD_{50}
P5-VJ-G	*innocua*	5.4	2.5	2.2	>3.2 x 10^8
LA-1	*innocua*	5.5	2.7	2.7	>3.0 x 10^8
SA3-VT	*innocua*	5.0	0	0.2	>2.5 x 10^8
SS-L-S	*innocua*	5.2	3.0	3.0	>6.5 x 10^8
LG5-LS	*innocua*	4.9	3.4	3.8	>2.7 x 10^8
H2-LG	*innocua*	5.2	2.7	2.5	1.0 x 10^8
SH3-VG-G	*innocua*	4.9	3.1	2.8	>2.8 x 10^8
		AVG 5.1	2.4	2.4	
KCL-1714	*ivanovii*	5.6	5.3	6.1	5.6 x 10^8
F-5999	*ivanovii*	5.6	5.2	6.0	8.2 x 10^8
		AVG 5.6	5.2	6.0	
F-4080	*seeligeri*	5.6	3.5	3.5	>1.6 x 10^8
GVL4-VS	*welshimeri*	5.2	2.3	2.3	>2.9 x 10^8
GVL4-LS	*welshimeri*	5.2	2.5	2.5	>3.1 x 10^8
H2-VJ-g	*welshimeri*	5.2	2.5	2.6	>3.4 x 10^8
CF1-VP	*welshimeri*	4.8	3.8	1.0	>5.0 x 10^8
		AVG 5.1	2.8	2.1	
GV2-VS	*monocytogenes*	5.2	4.5	5.1	2.9 x 10^1
S9-VJ-G	*monocytogenes*	5.6	4.5	5.0	1.4 x 10^3
H4-V-G	*monocytogenes*	5.4	4.7	5.3	1.0 x 10^2
Scott-A	*monocytogenes*	5.5	4.6	5.2	9.3 x 10^1
HO-VG-G	*monocytogenes*	5.5	4.7	5.4	3.1 x 10^1
MF2-L-P	*monocytogenes*	5.4	4.8	5.2	5.9 x 10^0
GVN4-VG	*monocytogenes*	5.3	4.6	5.2	3.1 x 10^3
GLB1-LS	*monocytogenes*	5.0	3.6	4.7	2.0 x 10^2
V3-VT	*monocytogenes*	5.0	4.5	5.2	1.3 x 10^1
LG4-VS	*monocytogenes*	5.0	4.3	5.1	4.2 x 10^1
GVG-VS	*monocytogenes*	4.9	3.8	4.6	1.1 x 10^2
CCR8-V-G	*monocytogenes*	5.4	4.7	5.5	1.0 x 10^3
F-4259	*monocytogenes*	5.4	4.5	5.2	2.0 x 10^3
F3-VJ-G	*monocytogenes*	5.1	4.8	5.3	3.1 x 10^1
VS2-VJ	*monocytogenes*	5.1	4.1	5.0	7.4 x 10^1
15313	*monocytogenes*	5.3	2.5	2.2	3.8 x 10^8
		AVG[b] 5.2	4.5	5.1	

[a]See text.
[b]Average does not include 15313, a nonhemolytic variant.

The LD_{50} values (Table 2) for hemolytic *L. monocytogenes* isolates ranged between 5.9 x 10^0 to 3.1 x 10^3 CFU. Mice infected with hemolytic *L. monocytogenes* died within 3 to 4 d postinfection. Nonvirulent *Listeria* were readily differentiable, with all *Listeria innocua, L. ivanovii, L. seeligeri,* and *L. welshimeri* strains having LD_{50} values $\geq 10^8$ CFU. A nonhemolytic, nonvirulent *L. monocytogenes* variant (15313) also had a $LD_{50} > 10^8$ CFU/

The ability of *Listeria* to enter and multiply within Hep-2 epithelial cells is summarized in Table 2. Because of cytotoxicity effects upon prolonged incubated (8-16 h), assays were limited to 3 h incubation with an MOI of 10:1. The kinetics of Hep-2 invasion (Fig. 1) were similar for all *L. monocytogenes,* being characterized by an initial attachment (T_0, intra and extracellular bacteria), invasion (T_1), and intracellular replication ($T_3 > T_1$). *L. ivanovii* isolates were more invasive than *L. monocytogenes* strains. The nonhemolytic variant strain of *L. monocytogenes,* as well as *L. innocua,* and *L. welshimeri,* were substantially less-invasive toward Hep-2 cells. *L. seeligeri* demonstrated an intermediate degree of internalization but did not replicate intracellularly.

The quantitative hemolytic activity of *L. monocytogenes* and *L. ivanovii* toward sheep red blood cells is shown in Table 3. Both activated and nonactivated culture filtrates of *L. ivanovii* gave the highest hemolytic titers. *L. monocytogenes* gave hemolytic titers in the range of 1:2 to 1:64 for activated filtrates and 1:1 and 1:16 for nonactivated culture filtrates. All other *Listeria* spp. were nonhemolytic. Scatterplots of hemolytic activity versus lethality

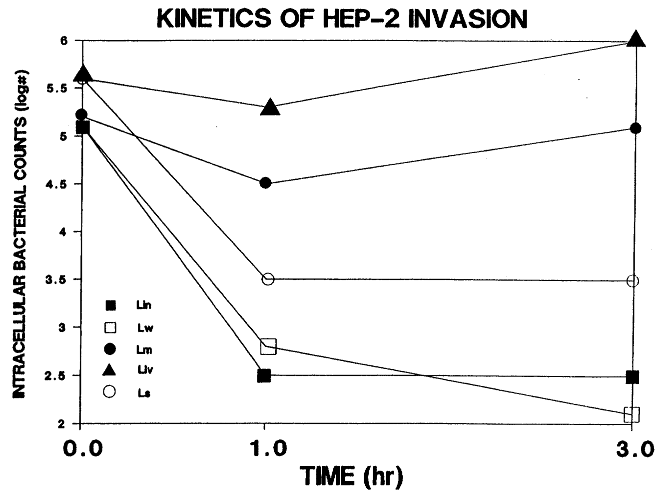

Figure 1. *Kinetics of Hep-2 cell invasion by various* Listeria *species.*
(Lin = *L. innocua*, Lw = *L. welshimeri*, Lm = *L. monocytogenes*, Liv = *L. ivanovii*, Ls = *L. seeligeri*).

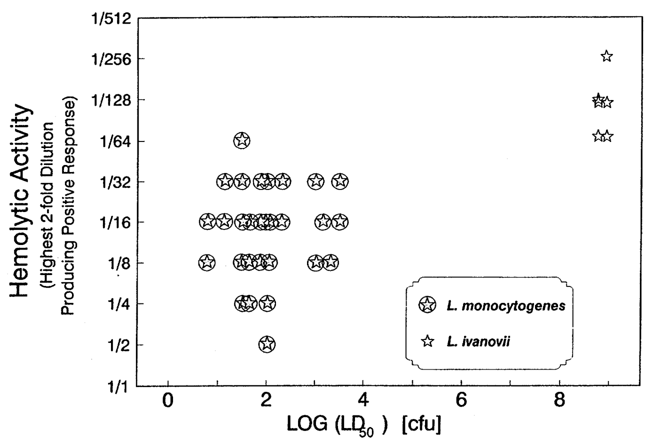

Figure 2. *Scatter plot depicting quantitative relationship between lethality to immunocompromised mice and hemolytic activity for hemolytic strains of* Listeria monocytogenes *and* Listeria ivanovii.

TABLE 3. *Hemolytic activity of* Listeria *spp. toward sheep red blood cells.*[a]

		Highest dilution producing hemolysis[b]					
		Activated			Nonactivated		
Strain	Species	1	2	3	1	2	3
KLC-1714	*ivanovii*	64	256	128	32	32	32
F-5999	*ivanovii*	64	256	128	32	32	ND[c]
GV2-VS	*monocytogenes*	32	64	8	16	16	1
S9-VJ-G	*monocytogenes*	16	16	16	4	2	1
H4-V-G	*monocytogenes*	8	2	4	2	1	0
Scott-A	*monocytogenes*	32	16	16	8	4	2
HO-VJ-G	*monocytogenes*	8	8	4	2	2	1
MF2-L-P	*monocytogenes*	16	16	8	8	4	2
GLB1-LS	*monocytogenes*	32	32	16	8	8	1
V3-VT	*monocytogenes*	32	32	16	16	16	4
LG4-VS	*monocytogenes*	8	16	4	4	4	0
GVG-VS	*monocytogenes*	8	16	8	4	4	0
CCR8-V-G	*monocytogenes*	32	8	32	4	1	4
F-4259	*monocytogenes*	8	8	8	1	2	1
F3-VJ-G	*monocytogenes*	16	16	8	4	2	1
VS2-VJ	*monocytogenes*	16	32	8	8	8	1

[a]*L. monocytogenes* ATCC 15313, a nonhemolytic variant, and all *L. innocua (8)* and *L. welshimeri (4)* isolates were hemolytic negative. The one isolate of *L. seeligeri* was hemolytic negative in this assay but did CAMP with *S. aureus*.
[b]Values represent results from three separate determinations performed on separate occasions.
[c]ND = not determined.

TABLE 4. *Cytotoxic effect of cell-free filtrates of* Listeria *spp. on CHO cells.*

		Highest dilution producing cytotoxic response[a]			
		Activated		Nonactivated	
Strain	Species	+ Serum[b]	- Serum	+ Serum	- Serum
KC1-1714	*ivanovii*	1:4	1:32	1:2	1:16
F-5999	*ivanovii*	1:8	1:32	1:2	1:16
S9-VJ-G	*monocytogenes*	1:2	1:2	1:2	1:2
H4-VJ-G	*monocytogenes*	1:2	1:2	1:2	1:2
Scott-A	*monocytogenes*	1:2	1:2	1:2	1:2
HO-VJ-G	*monocytogenes*	1:2	1:2	1:2	1:2
MF2-L-P	*monocytogenes*	1:2	1:2	1:2	1:2
GVN4-VG	*monocytogenes*	1:2	1:2	1:2	1:2
GLB1-LS	*monocytogenes*	1:2	1:2	1:2	1:2
V3-VT	*monocytogenes*	1:2	1:2	1:2	1:2
LG4-VS	*monocytogenes*	1:2	1:2	1:2	1:2
GVG-VS	*monocytogenes*	1:2	1:2	1:2	1:2
CCR8-VG	*monocytogenes*	1:2	1:4	1:2	1:4
F-4259	*monocytogenes*	1:2	1:4	1:2	1:2
F3-VJ-G	*monocytogenes*	1:2	1:4	1:2	1:2
VS2-VJ	*monocytogenes*	1:2	1:2	1:2	1:2

[a]*L. monocytogenes* ATCC 15313, *L. seeligeri*, and all *L. innocua*, *L. welshimeri* were cytoxic negative. Assays replicated on two separate occasions.
[b]10% fetal bovine serum.

to immunocompromised mice (Fig. 2) were indicative of a lack of quantitative relationship between these virulence markers.

Culture filtrates of *L. ivanovii* were the most cytotoxic (Table 4) toward CHO cells, while *L. monocytogenes* gave low level cytotoxic responses (average cytotoxic value of 1:2) with both activated and nonactivated culture filtrates. Other *Listeria* spp. were consistently noncytotoxic, including the nonhemolytic variant of *L. monocytogenes* (ATCC 15313).

Transmission electron microscopy revealed both *L. monocytogenes* (Scott-A) and *L. ivanovii* (F-5999) within several cytoplasmic compartments devoid of an endosomal membrane (Fig. 3A, arrow), while *L. innocua* (H2-LG) was not observed intracellularly. Due to the lengthy incubation period (3 h), attachment and pseudopod formation were not observed; however, a few cells were wedged between the microvilli. In several instances hemolytic *Listeria* (*L. monocytogenes* and *L. ivanovii*) were observed in what appeared to be stages of division (fission) within the cytoplasm, with *L. ivanovii* showing a considerably higher number of intracellular bacteria.

Intracellular *Listeria* were often observed surrounded by a fine filamentous matrix with no surrounding mem-

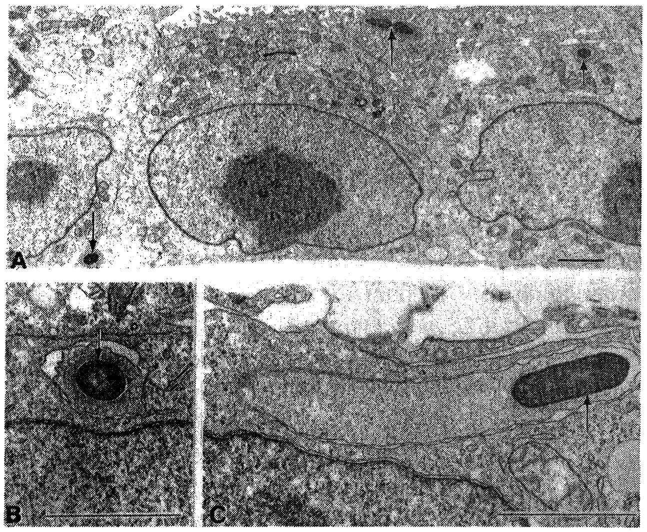

Figure 3. *Thin section of Hep-2 cell monolayer infected with* L. monocytogenes *(Scott-A). (A) Four bacterial cells are located within the cytoplasm (arrows). Portions of Hep-2 cells are shown with intracellular* Listeria *in membrane bound inclusions (arrows) in cross section (B), and longitudinal section (C). Bar = 1 μm.*

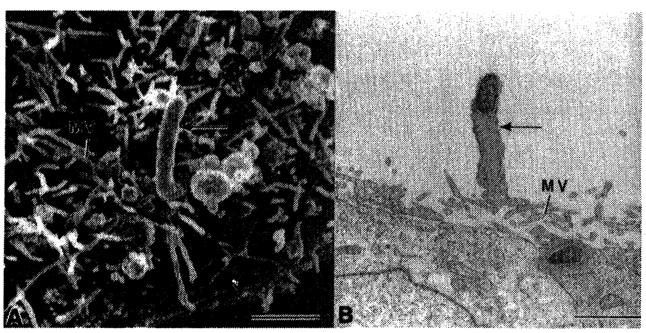

Figure 4. *"Finger-like" projections of infected Hep-2 cells. (A) Scanning electron micrograph of Hep-2 cell surface showing a projection (arrow) among numerous microvilli (MV), and (B) a thin section of a projection (arrow) showing a single* L. monocytogenes *cell at the tip among microvilli (MV). Bar = 1 μm.*

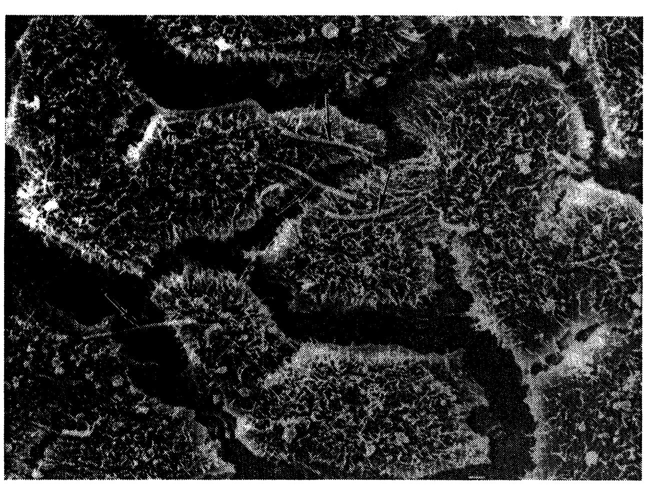

Figure 5. *Scanning electron micrograph of the surface of a monolayer of Hep-2 cells following infection with* L. monocytogenes. *Several "finger-like" projections (arrows) extend between neighboring cells among the numerous microvilli. Bar = 1 μm.*

brane or coated with a matrix within a membrane bound inclusion (Fig. 3B & C). These filamentous matrices formed "finger-like" projections with a bacteria cell at the apical end (Fig. 4A & B). Images of the surface of the cell monolayer revealed these projections extending from one Hep-2 cell towards a neighboring one (Fig. 5). The size of the projections was around 500 nm in diameter and several micrometers in length. Phase contrast micrographs of infected monolayers depict the "finger-like" projections extending extracellularly (Fig. 6A). The fluorescent probe, phalloidin, labeled these projections indicating that these projections contained actin microfilaments. The actin was observed surrounding the bacteria and extending extracellularly (Fig. 6B). *Listeria* appear to be able to utilize

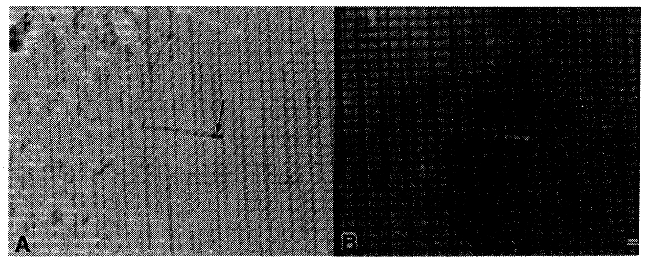

Figure 6. *Correlated optical phase contrast (A) and epifluorescence (B) micrographs of an infected Hep-2 cell showing a "finger-like' projection containing a single* Listeria *(arrow) at the tip. The distribution of fluorescent phalloidin along the projection indicating the distribution of F-actin is illustrated in (B). Bar = 1 μm.*

cytoskeletal actin to infect adjacent cells (Fig. 5). Preliminary observations suggested that actin microfilament projections were less abundant with *L. ivanovii* infected monolayers than with *L. monocytogenes*).

DISCUSSION

Macrophages play a key role in defense intracellular pathogens such as *L. monocytogenes* (*21,23*). Depletion of nonimmune resident phagocytes from mice made them highly susceptible to infection by strains of hemolytic *L. monocytogenes,* but the animals retained their resistance to other species of genus including *L. ivanovii*. Treatment with carrageenan results in the inactivation of approximately one-fourth of the macrophage series (*33*), allowing virulent *L. monocytogenes* to multiply unrestricted. Although drastic in nature, this mouse model closely mimics a portion of the cases observed in humans (*31*) and allows a clear differentiation of virulent species. Some degree of differences in virulence among strains of *L. monocytogenes* was observed, agreeing with the observations of Hof and Hefner (*13*).

Several investigations have shown that hemolysin is an important factor (*5,15*). Qualitatively, we observed a strong relationship between hemolytic activity and virulence in *L. monocytogenes* (but not *L. ivanovii*). However, like Kathariou et al. (*14*), a quantitative correlation between hemolysin production and lethality was not evident (Fig. 2). *L. ivanovii* was highly hemolytic but nonvirulent for mice, suggesting that either the two species produce distinctly different classes of hemolysins or there are additional postinvasive virulence determinants in *L. monocytogenes* that act in concert with the hemolysin(s). The latter seems more likely based on the relatedness of hemolysin and invasiveness assays. Kreft et al. (*16*) recently observed that both *L. monocytogenes* and *L. ivanovii* strains produce 58 KD sulfhydryl-activated cytolysins with a high degree of homology, suggesting that other unknown factors in *L. monocytogenes* may act in concert with listeriolysin O.

Hemolytic *L. monocytogenes* and *L. ivanovii* were able to adhere, invade, and proliferate intracellularly, whereas nonhemolytic *Listeria* spp. (*L. innocua, L. welshimeri*) were largely restricted to adherence. Khun et al. (*18*) concluded that the extracellular hemolysin (listeriolysin O) in *L. monocytogenes* was not involved in the initial entry of this bacterium to 3T6 mouse embryo fibroblast since both hemolytic and nonhemolytic derivatives (Tn-916 transposon conjugates) were taken up by 3T6 fibroblasts, with equal high efficiency. Similarly, Gaillard et al. (*6*) and Portnoy et al. (*25*) observed that listeriolysin O was not involved in the process of initial internalization (invasiveness), since nonhemolytic *Listeria* was able to invade both Caco-2 and Henle-407 epithelial cells at the same rate as the hemolytic revertant or parent strain. Kuhn et al. (*18*) observed that other *Listeria* spp., including the highly hemolytic *L. ivanovii,* were not taken up by 3T6 cells. This difference in uptake with *L. ivanovii* by Hep-2 and 3T6 cells could be due to the nature of the cells, one being epithelial-like and the other fibroblastic.

Our observations suggest that there are additional postinvasive factors that account for the difference in virulence between *L. monocytogenes* and *L. ivanovii*. One postinvasive factor shown with hemolytic strains of *L. monocytogenes* and not *L. ivanovii* is their ability to disrupt the phagosomal membrane of phagocytes by irreversible binding to the membrane's cholesterol (*7,17*) thereby escaping the microbiocidal mechanisms (*1*).

Qualitatively, there was a high degree of relatedness between hemolytic activity and cytotoxicity. However, the lack of quantitative correlation within the hemolytic *L. monocytogenes* plus the lack of increased cytotoxicity upon activation with *L. monocytogenes* (but positive activation with *L. ivanovii*) suggests that the hemolysin and the cytotoxin may be separate agents in *L. monocytogenes*. Recently, Farber and Spiers (*3*) were able to obtain identical titers in both hemolytic and cytotoxic assays. This similarity was obtained only by adjusting cell densities, indicating that cell-free culture supernatants appear to have different affinities toward sheep red blood cells and CHO cells. Further research is needed to establish definitive proof.

The formation of actin microfilaments by *L. monocytogenes* in cultured cells has also been noted by Tilney and Portnoy (*34*) and Mounier et al. (*22*). Tilney and Portnoy (*34*) proposed an infection cycle wherein *L. monocytogenes* passes from cell to cell without exposure to the extracellular environment. Mounier et al. (*22*) concluded that intracellular *L. monocytogenes* are enveloped with a thick layer of F-actin, and that hemolysin-mediated lysis of the phagocytic vacuole and subsequent interaction with host cell microfilaments may represent a major virulence factor. The current study extends those observations by inclusion of *L. ivanovii* which also induced formation of actin microfilaments in cultured cells. Further, the identification of microfilaments in a third cell line suggests that this is a characteristic aspect of intracellular growth by *L. monocytogenes*.

Our preliminary observations suggest that although *L. ivanovii* is more invasive in vitro than *L. monocytogenes,* the nonpathogenic species appears to induce microfilament projections to a lesser extent than the pathogen. This observation may account for the large difference in virulence between *L. monocytogenes* and *L. ivanovii*. Further investigations are underway to determine the implications of this observation and if cellular actin is induced within the tissues of a live host infected with *L. monocytogenes*.

No systematic differences were detected between the clinical and food isolates of *L. monocytogenes*. Our results support those of Hof and Hefner (*13*) that all *Listeria* spp. other than *L. monocytogenes* are avirulent. Further, the results of the current study suggest that until additional virulence determinants are identified, all phenotypically normal (i.e., hemolytic) *L. monocytogenes* strains from food sources should be considered potentially pathogenic.

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